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LIST OF ABBREVIATIONS

˚C	Degree Celsius
µg	Microgram
µl	Microliter
µm	Micrometre
µM	Micromolar
ATCC	American Cell Type Collection
BPE	Bee pollen extract
GA	Gallic acid
L929	Normal mouse fibroblast cell
MCF7	Human breast cancer cell line
kPA	Kilopascal
FBS	Fetal Bovine Serum
RPMI	Roswell Park Memorial Institute
CO_2	Carbon dioxide
g	Gram
IC_{50}	50% inhibitory concentration
EC_{50}	Effective concentration
mg	Milligram
ml	Millilitre
nm	Nanometre
L	Litre
PBS	Phosphate buffer saline
PI	Propidium iodide
v/v	Volume / volume
USA	United States of America
UK	United Kingdom
TCA	Trichloroacetic Acid

SEM	Standard error mean
OD	Optical density
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

AKTIVITI ANTIPROLIFERATIF EKSTRAK DEBUNGA LEBAH KELULUT KEATAS KANSER PAYUDARA MCF-7

ABSTRAK

Tujuan kajian ini adalah untuk menentukan aktiviti antioksidan dan antiproliferatif daripada ekstrak debunga lebah kelulut spesis *L. terminata*. Aktiviti antioksidan ditentukan menggunakan 2,2-diphenyl-2-picrylhydrazyl (DPPH), dan Folin-Ciocalteu telah digunakan untuk menguji jumlah kandungan fenolik dalam ekstrak debunga lebah. 3-[4,5-dimethylthiazol-2-YL] -2,5-diphenyltetrazolium (MTT) telah digunakan untuk menentukan aktiviti antiproliferatif. Kepekatan efektif (EC50) ekstrak debunga kelulut adalah 0.36mg/ml. Jumlah ekstrak fenolik pada 20mg /ml ekstrak debunga lebah adalah 34.93±0.150mg/g. MTT menunjukkan ekstrak debunga lebah mempunyai kepekatan perencat (IC50) 25.5mg/ml bagi sel normal L929 manakala IC50 untuk sel kanser payudara MCF-7 adalah 15mg/ml, kajian ini menunjukkan bahawa ekstrak debunga lebah mempunyai kesan sitotoksik dalam sel-sel kanser, tetapi tidak dalam sel-sel normal. Gabungan cisplatin dan ekstrak debunga lebah menunjukkan interaksi sinergi dalam mendorong aktiviti pembunuhan sel kanser payudara MCF-7.

ANTIPROLIFERATIVE ACTIVITY OF STINGLESS BEE POLLEN EXTRACT ON HUMAN BREAST CANCER CELL LINE MCF-7

ABSTRACT

The aims of this study were to determine the antioxidant and antiproliferative activity of the bee pollen extract from stingless bee *L. terminata*. Antioxidant activity was determined using 2,2-diphenyl-2-picrylhydrazyl (DPPH), and Folin-Ciocalteu assays was used to test the total phenolic content in the bee pollen extract, the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) assay was used to determine antiproliferative. The effective dose (EC_{50}) of bee pollen extract was 0.36 mg/ml. Total phenolic content of bee pollen extract at 20mg/ml of bee pollen extract was 34.93 ± 0.150mg/g.The MTT assay showed that bee pollen extract median inhibitory concentration (IC_{50}) 25.5mg/ml for normal cell line L929 while IC_{50} for breast cancer cell MCF7 was 15mg/ml, this study indicates that bee pollen extracts have cytotoxic effect in cancer cells, but not in normal cells. The combination of cisplatin and bee pollen extract showed synergistic interaction in inducing the killing activity of breast cancer cell line MCF7.

CHAPTER 1: INTRODUCTION

Cancer is known as malignant tumor which this multifactorial cell disease involved abnormal cellular proliferation (Neve *et al.*, 2006). The development of cancer cell depends on various changes of genetic and epigenetic in the cell (Giri *et al.*, 2006), uncontrollable proliferation and lack of apoptosis event (Weaver and Cleveland, 2005). The development of cancer usually due to the imbalance expression of oncogenes, tumor suppressor gene and the alteration of the microRNA (Yanaihara *et al.*, 2006). Treatment and cure of cancer problem was scientifically challenging and gave a serious burden on public health system which claim nine million lives worldwide by 2015 (Rajesh *et al.*, 2011). In Malaysia, cancer was established as the fourth main cause of death after cardiovascular disease (Lim, 2002). The death from breast cancer was reported by The Ministry of Health Malaysia to be rank among the top 10 cancer-related deaths in the country (Yip *et al.*, 2006). As the cancer becomes one of the life threatening diseases throughout the world, there are interest to develop a new anticancer drugs, which are more specific and less side effects than chemotherapeutic drugs. Anticancer drugs have distinct mechanisms of action which may vary in their effects on different types of normal and cancer cell (Pascoal *et al.*, 2014). There was plenty of antineoplastic agents that widely used for treatment of malignant cancer, but these anticancer drugs exhibit genotoxic and cytotoxic effect which inhibits the growth (Sánchez-Suárez *et al.*, 2008). Presently, about 65% of the nature based chemotherapeutic drugs were established and have a long history in both traditional and modern cancer treatments (Conforti *et al.*, 2008).

The relationship between bees, flowers and man is one of the wonders of the universe, being a living proof that the flora, fauna and man were made to live in harmony. Bees need flowers to feed themselves, plants need bees to be pollinated and to produce seed to ensure the perpetuation of plant species (Almeida-Muradian *et al.,* 2005; Pascoal *et al.,* 2014). Bee pollen was originated from the agglutination of flower pollens with nectar and salivary substances of the bees. Bee pollen is known and used since ancient times (Pratsinis *et al.,* 2010; Kustiawan *et al.*, 2014). Bee pollen is also the only perfect and complete food (Almeida-Muradian *et al.*, 2005). It consists of polyphenol substances that are very useful in traditional and conventional medicine (Kustiawan *et al.*). Polyphenol known to help reduce the risk of severe diseases and cancer due to its antioxidant, antimutagenic, anti-inflammatory, antibacterial and anticancer activity (Jaganathan and Mandal, 2009; Pratsinis *et al.*, 2010; Kustiawan *et al.*, 2014; Pascoal *et al.*, 2014). Thus, it is possible that bee pollen can serve as potential sources for developing new drugs and more effective anti-cancer agents for future therapy (Wu and Lou, 2007).

OBJECTIVES :

- To study the antioxidant activity of stingless bee pollen extract.
- To study the antiproliferative activity of bee pollen extract on human breast cancer cell line MCF-7
- To explore the combination effect of bee pollen extract and anticancer drug cisplastin [cis-diammine-dichloroplatinum (II)] on breast cancer cell line (MCF-7)

HYPOTHESIS:

Bee pollen extract with good antioxidant activity has antiproliferative activity towards human breast cancer cell line (MCF-7), and its anti-proliferative activity exhibits a synergistic effect with anticancer drug cisplastin [cis-diammine-dichloroplatinum (II)].

CHAPTER 2: LITERATURE REVIEW

2.1 Breast cancer

2.1.1 Prevalence and risk factor

The breast is made up of glands lobules that produced milk and consists of thin tubes ducts that transported the milk from the lobules to the nipple. Breast tissue contains fat and connective tissue, lymph nodes, and blood vessels. Mammary carcinoma, the most common type of breast cancer is ductal carcinoma, which primarily developed in the cells of the ducts (Yip *et al.*, 2006). Breast cancer proliferates in the cells of the lobules and metastasizes into the tissues in the breast. Invasive breast cancer is cancer that has spread from where it began in the ducts or lobules to surrounding tissue (Hisham and Yip, 2004). National Cancer Registry of Malaysia in 2006 reported that breast cancer one of the most diagnosed cancer among Malaysian women, which about 30.4 % cases reported without regard age and ethnicity (Yip *et al.*, 2006). Different type of cancer showed different risk factors such as improper diet, genetic potentiality, and surrounding environmental factors (Are and Shaha, 2006). Breast cancer usually caused by single or combination of external and internal factors such as age and female sex as at the age of woman between 35 to 39 years showed higher rate of incidence (Lim, 2002). Breast cancer also a type of cancer which interrelated with the cumulative lifetime exposure of estrogen hormone in the body, and this was one of the reason why the chances of women getting breast cancer was higher than men (Vona-Davis and Rose, 2009). About 5-10% breast cancer are due to genetic inheritance (Synowiec *et al.*, 2008). Other factor that related to the risk of breast cancer included high mammographic breast density, the used of exogenous hormones such as therapy that involved with hormone replacement, oral contraceptives, obesity and the

consumption of alcohol in daily life (Dumitrescu and Cotarla, 2005;Synowiec *et al.*, 2008).

2.1.2 Breast cancer classification and treatment

Breast cancer are categorized into different subgroup according to the histological type of the grade of tumor, the stage, lymph node status and molecular profile (Dumitrescu and Cotarla, 2005). The diagnosis and the treatment of breast cancer became more challenging when it involved the heterogeneity of the breast cancer (Saltz *et al.*, 2008;Holliday and Speirs, 2011). There were few options for breast cancer such as surgery, chemotherapy, radiotherapy and hormonal therapy), however most of the treatment was very high in cost and at the same time gave adverse effect to the patient which also cause the resistance of cancerous cell towards the treatment (Alwan, 2011).

2.2 Chemotherapeutic agents

Chemotherapy is known to be one of the most acceptable treatments worldwide. Chemotherapeutic agents were varied in their target and effectively kill most of the cancer cells. It can be considered as effective treatments in endocrine-responsive disease, however less effective for endocrine unresponsive tumors (Shaked *et al.*, 2008). There are many types of strong potential chemotherapeutic agents that provided many different ways of killing the cancerous cell had been developed (Risques *et al.*, 2011). However, chemotherapeutic agents alone sometime doesn’t give an increment in cure rates of cancer cells (HemaIswarya and Doble, 2006; Hemaiswarya *et al.*, 2008). Chemotherapeutic treatment can cause side effects which can be divided into short-term and long term effects. Short term effects usually occurs during the period of treatment and the therapy will complete within a month, while the long term effect occurred post

treatment and long lasting for many years (Burstein *et al.*, 2001). Examples of some side effects listed in table 2.1.

Table 2.1 : List of reported short term and long term effects of chemotherapeutic agent (Esserman et al., 2001)

Short term effect	Long term effect
- Emesis - Nausea - Stomatitis - Myalgias - Hot flashes - Neuropathy - Fatigue - Myelosuppression - Thromboembolism - Myelosuppresion - Alopecia	- Premature Menopause - Weight gain - Cardiac dysfunction - Leukemia - Cataracts - Vascular related thrombotic events - Development of another cancer

More than 90% of patient with metastatic cancer was accounted for the failure of the treatment due to the resistance of cancer cell toward chemotherapeutic agent, as chemotherapeutic agent was not sensitive to cancer cell (Longley and Johnston, 2005). Chemotherapeutic resistance could happen due to internal exposure which known to be intrinsic resistance, and external exposure which was extrinsic resistance (Scotto, 2003). The resistance of cancer cell toward chemotherapeutic drugs due to a number of mechanism of cancer cell resistance such as defects in DNA repair pathway, suppression of cell death pathway, exclusion or active export of drug from the cell and regulation of epigenetic (Dingli and Michor, 2006).

2.2.1 Cisplatin (cis-diamminedichloroplatinum(II)) (CDDP)

Cisplatin is a chemodrug that interrupt the growth of cancer cells and inhibit their growth from spread throughout body. This antineoplastic agent was widely used to treat malignant solid tumor including testicular, ovarian, breast, lung, neck, head, bladder cancer (Aebi *et al.*, 1996). Cisplatin is an effective antitumor medicine that often combined with other cancer drugs (Abdella *et al.*, 2009). The type and extent of a cancer determines how effectively this medicine slows or stops the growth of cancer cells in the body. Majority of antineoplastic drug has generic growth property, display genotoxicity, and cytotoxic effect which could inhibit the growth of the cancer cell, but the toxic effect of the drug could also lead to initiation of unrelated tumor (Abdella *et al.*, 2009). Cisplastin also has severe toxic effect which inhibit the therapeutic efficacy such as bone marrow toxicity, neurotoxicity, nephrotoxicity, hepatotoxicity after undergo chemotherapy (Arany *et al.*, 2004; Chandrasekar *et al.*, 2006; Abdella *et al.*, 2009). Cisplatin required dose administration and restriction for it optimal activity in cancer chemotherapy (Arany *et al.*, 2004).

2.3 Natural products as a drug

A chemical compound or substances that formed by living organism that showed biological and pharmacology activities are defined as natural products (S Gollahon *et al.*, 2011). About 70% to 80% of herbal products are an essential source of medicine (Organization, 2002). Although there was development in anticancer drug, the natural source products still remained as products. Natural product was affordable and at the same time they are finer in term of safety and efficacy compared to synthetic products with less side effects (Johnston *et al.*, 2006; Demain and Sanchez, 2009). Unfortunately, some of the natural products proclaimed for their advantage in traditional used were not scientifically proven for efficacy and adverse effects (Koehn and Carter, 2005). Natural products that had biological and pharmacology activities usually make them vital sources for drug discovery (Cragg *et al.*, 1997). Around year 2000-2005 there were about 23 new drugs were identified from natural sources which can act as an ailment to various diseases such as cancer, cardiovascular, metabolic diseases and many others (Koehn and Carter, 2005; S Gollahon *et al.*, 2011).

Malaysia was known to be one of the countries that have variety natural products from tropical rainforest. The abundance of natural sources in Malaysia give many potential to researchers to develop medicinal products and into commercial market, at the same time coordinate research and development activities on medicinal plants was set up and formed in 1994 by The Malaysian Natural Products Society (Muhammad and Awaisu, 2008) to further promote our natural products. Over than 1300 species of natural sources that comes from tropical rainforest of Malaysia showed medicinal properties such as *Catharanthus roseus,* which showed ability to become an anticancer drug and propolis from stingless bee, which showed antiproliferative activity

on human cancer cell lines (Chandrasekar *et al.,* 2006). Whereas, anticancer activity of of *Strobilanthes crispus* known as "Pecah Beling"were seen on human breast and prostate cancer cells in vitro (Yaacob *et al.*, 2010; Mohamad *et al.*, 2011).

2.4 Natural product as adjuvant therapy

The effects of natural product in combination with anticancer drugs was investigated for their potential in improving the killing effects of multidrug resistance cancerous cell, and at the same time can overcome the toxicity effect against normal cell (Da Rocha *et al.*, 2001; Butler, 2004). Some compounds in natural product showed a great combination effects which may work synergistically or antagonistically with the therapeutic activity of drugs (Hemaiswarya *et al.*, 2008). For example, curcumin have antiproliferative effects in combination with doxorobucin in treating breast cancer cell (Hosseinzadeh *et al.*, 2011) and at the same time, curcumin also give a great effect of antitumor and apoptotic activities of cisplatin against ovarian cell (Chan *et al.*, 2003). Another example of component that derived from natural sources is genistein which worked synergistically with tamoxifen against breast cancer (HemaIswarya and Doble, 2006). Oriental herbal medicines also showed anti-tumor effect and showed potential to overcome the side effect to normal cell (Kang *et al.*, 2006). For example Bojungbangdocktang known as a Korean herbal medicine has ability to protect non-cancerous epithelial cells of the breast (MCF10A) against apoptosis and cytotoxicity induced by cisplatin (Kang *et al.*, 2006) and improved the anticancer effect of cisplatin against breast cancer cell MCF-7 (Fan *et al.,* 1995).

2.5 Bee pollen

2.5.1 Definition and characteristics of stingless bee pollen

Bee pollen known as a pollen ball from the male seed of flower that gathered and packed by worker of stingless bees into pellets then transfer them back to colony by attached at their corbiculae (hind leg) of the bees (Almeida-Muradian *et al.*, 2005). Bee pollen is a combination with plant nectar and bee saliva which contain its own digestive enzyme secretion from the hypopharyngeal glands, such as α and β-glycosidase enzymes (Tavdidishvili *et al.*, 2014). Bee pollen also known to be the primary source of protein in the bee hive as the bees consumes as their own diets and at the same time they fed their own larvae. (Carpes *et al.*, 2007). This meliponiculture product is known as product that are gathered and stored in honeycomb and covered the pollen with honey (Leja *et al.*, 2007). Bee pollen was rich in minerals, simple sugars, proteins, vitamins, fatty acid, flavonoids, phenolic acid, carotenoids, phytosterols and other phytochemicals that responsible for showing the antioxidants activity of the bee pollen (Silva *et al.*, 2006). The composition of the bee pollen might vary depending on the floral, geographical area of the bee pollen, the season and method used to collect the bee pollen, as well as storage of the bee pollen after harvested (Cook *et al.*, 2003; Campos *et al.*, 2008). These factors might also affect the physical properties of the bee pollen such as color, shape, quantity. Hence, most of the reported bee pollen showed different medicinal properties (Carpes *et al.*, 2007).

2.5.2 Benefits of bee pollen

Bee pollen known to be used as a source of energy and nutrients for human to consume (Serra Bonvehi and Escolà Jordà, 1997). Bee pollen known worldwide for its used for dietary purpose and food supplement and has the ability to serve several pharmacological effects. However at the moment, there was not enough evidence to support on the relationship between chemical constituents with its pharmacological effects (Mason, 2012). Bee pollen also categorized under nutraceuticals and therapeutic food that provides nutrients to reduce the potential in developing certain illness. This is because bee pollen has very high in amino acids and at the same time also is high in various vitamins and minerals, such as calcium and lysine, as well as phosphorus. Lysine has a potential to absorb calcium, which proven that potential for bone health and it can help with the formation of bone callus that is associated with fractures (Hayden, 1980). It is also help to improve the bone health which is very suitable for the brittle bones (Yamaguchi et al., 2006). Bee pollen also beneficial in preventing osteoporosis (Hamamoto *et al.*, 2006).

The therapeutic properties of bee pollen include anti-inflammatory, antibacterial, antifungal, wound healing, cytotoxicity effect, immune booster and at the same time stimulate the tissue regeneration (Šarić *et al.*, 2009). It is also been used for treatment of acute and chronic free radical-mediated diseases such as artherosclerosis, diabetes and cancer (Wu and Lou, 2007). The therapeutic properties of bee pollen able to overcome multi-factorial causes of carcinogenesis such as free radicals, chronic infection, inflammation and low immune status (Medeiros *et al.*, 2008).

Several studies shown that bee pollen had a great potential to sustain antioxidant capacity due to high content of phenolic acids and flavonoid content (Kustiawan *et al.*, 2014). Recent findings also proven that bee pollen of stingless bee showed cytotoxity effect toward human cancer cell line including ductal carcinoma, lung undifferentiated cancer, liver hepatoblastoma, and breast cancer (Chantarudee *et al.*, 2012; Kustiawan *et al.*, 2014). The increasing data collected on stingless bee pollen for medicinal and therapeutic purposes led to the resurgence of interest in stingless bee pollen.

CHAPTER 3: MATERIALS AND METHODS

3.1 Sample collection

Bee pollen was harvested from the stingless bee hive located at Tasek Gelugor, Penang. Bee pollen sample was from the species *L. terminata*, which was identified by Entomology unit, MARDI, Serdang. Harvested bee pollen was then dried at 40℃ for a week before kept in +4℃ fridge until further used.

3.2 Extraction of bee pollen

This method was modified from (Kustiawan *et al.*, 2014).Ten grams of bee pollen sample was weighed and put into 50ml Falcon tube. Twenty five millilitre of methanol was added. Three different concentrations of methanol (70%, 80% and 100%) were used for bee pollen extraction. The mixture of bee pollen with methanol was then vortexed for 10 minutes, and sonicated for 1 hour in 4℃ . The bee pollen mixture was then kept overnight at +4℃ .

The mixture was then centrifuge at 6000 rpm for 10 minutes and the supernatant was transferred into the clean falcon tube. The remaining precipitate was discarded. These processes were repeated until the supernatant become clear. The supernatant was then filtered using 0.2µm sterile filter unit (Millipore, USA). The supernatant was dried in rotary evaporator at 40℃ and freeze dried for one week. After one week the sample was weighed again and kept in -20℃ until further use. The percentage of extraction efficiency was calculated using formula:

$$\% \text{ extraction efficiency} = (M_o / M) \times 100$$

* M_o: weight of freeze dried bee pollen extract, Weight of bee pollen before extract

3.3 2,2-Diphenyl-1-picrylhydrazyl (DPPH) antioxidant activity of bee pollen extract

This method was modified from (Kustiawan *et al.*, 2014). The stock solution of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was prepared using 12mg of DPPH powder (Sigma Aldrich, USA) with 50ml of methanol in dark. Trolox (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) (Sigma Aldrich, USA) was used as standard to generate standard curve with concentration 25µM, 169µM, 313µM, 475µM, and 600µM. About 7.5µl of trolox from each concentration was pipetted with 150µl of 270 µM DPPH. Absorbance was read using microplate reader (BMG Labtech, Germany) at 517nm after 30 minutes. The bee pollen samples stock were prepared by diluting 0.1g of bee pollen extract with 1ml of methanol and diluted into concentrations of 2.5mg/ml, 5mg/ml, 10mg/ml, 15mg/ml, and 20mg/ml. About 7.5µl of bee pollen extract from each concentration was mixed with 150µl of 270µM DPPH, and the mixture's absorbance was read using microplate reader. For the negative control, methanol without BPE was used. (Kustiawan *et al.*, 2014). DPPH free radical scavenging activity was calculated using the following formula:

% scavenging = [Absorbance of control - Absorbance of test sample/Absorbance of control] X 100

3.4 Total phenolic content of bee pollen extract

This method was modified from (Chan *et al.*, 2008).The phenolic content of bee pollen extracted was tested with different concentrations which were 2.5mg/ml, 5mg/ml, 10mg/ml, 15mg/ml, and 20mg/ml. Folin-Ciocalteau solution was prepared by adding 2.5ml Folin-Ciocalteau reagent with 25ml distilled water. Sodium carbonate solution was prepared using 1.5g of sodium carbonate powder mixed with 20ml distilled water. About 12µl of bee pollen extract from each concentration was mixed with the freshly prepared 60µl Folin-Ciocalteau solution, then left for 10 minutes and the mixture was resuspended. Then, 48µl sodium carbonate was added and the mixture resuspended at 37℃ for 30 minutes. The absorbance was read at 760nm. Gallic acid was used as standard in this experiment. Gallic acid stock was prepared 20µM and diluted with concentrations of 0.125mM, 1.25mM, 2.5mM, 5mM, 10mM, and 20mM were used to construct a calibration curve. Distilled water was used for negative control. All samples and control were prepared triplicates. The total phenolic content of the samples were analysed based on the measured absorbance, the concentration of phenolics was read in (mg/ml) from the calibration curveline, the total phenolic content the bee pollen extracts was expressed in the terms of gallic acid equivalent (mg of GA/g of extract).

3.5 Cell culture

3.5.1 Maintenance of cell lines

Two types of cell lines were used in this study which were normal mouse fibroblast cell L929 and breast cancer epithelial cell MCF-7.

3.5.2 Reagents for cell culture

The reagents used in this study were Dulbecco's modified Eagle Medium (DMEM) (Gibco BRL, UK) for MCF-7 and L929, FBS (100ml) (Gibco BRL, UK) penicillin/streptomycin solution 10mg/ml (Gibco BRL, UK) 1X phosphate buffered saline (Gibco BRL,UK) and Trypsin 0.25%, EDTA 0.03% (Gibco BRL, UK). All reagents were thawed and aliquot into small sterile tubes and kept -20℃ until further used.

3.5.2 (a) Medium

One type of media reagents used in this study was Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL,UK) for MCF-7 and L929 cells.

3.5.2 (b)Heat inactivated Foetal Bovine Serum (FBS)

A bottle of readymade FBS (100ml) (Gibco BRL,UK) was heat inactivated by placing it in water bath at 56℃ for 30 minutes. The serum was then aliquoted into sterile 15ml Falcon tubes and kept at -20℃ for further used.

3.5.2(c) Penicillin/ Streptomycin stock solution

A bottle of ready-made penicillin/streptomycin solution 10mg/ml (Gibco BRL, UK) was thawed and aliquoted into sterile 5ml eppendorf tube and kept -20℃.

3.5.2(d) 1x Phosphate Buffered Saline (PBS)

A bottle of ready-made 11X phosphate buffered saline (Gibco BRL,UK) and was aliquoted 50ml in falcon tube and kept in 4℃ for further used.

3.5.2(e) Trypsin 0.25%, EDTA 0.03 %

Ready-made Trypsin 0.25%, EDTA 0.03% (Gibco BRL, UK). All reagents were thawed and aliquot into small sterile tubes sterile 15ml tubes and kept -20℃ for further used.

3.5.2(f) Complete growth medium for MCF-7 and L929 cells

MCF-7 and L929 was cultured and maintained at 37℃ in 5% CO_2 humidified atmosphere in DMEM medium supplemented with 5% FBS and 1% antibiotic (penicillin/streptomycin).

3.5.2(g) Cryopectant Medium

MCF-7 and L929 cells were cryopreserve in 10% dimethyl sulphoxide (DMSO) in the cryotube and stored in -80℃ for further used.

3.5.3 Cultures procedures and conditions

All the procedures were carried out inside the class II Biohazard Cabinet and handled with proper aseptic technique to avoid contamination. Good cell practice guidelines were practiced throughout this study.

3.5.3(a) Retrieving of cells from frozen storage

A cryovial containing frozen cells was retrieved from -80℃ freezer and left at room temperature for about 5 minutes to thaw the cells. Thawed cells were then transferred into a sterile 15ml falcon tube containing pre-warmed growth medium. It was then centrifuge at 1000rpm for 5 minutes. The supernatant was discarded and 1ml of PBS was added, before centrifuged again at 1000rpm for 5 minutes. This procedure was done to reassure the complete removal of cryopectant medium from the cells. The supernatant was discarded and the cell pellet was resuspended in 1ml growth medium. The cell suspension was divided into two 25cm^2 culture flasks, containing 5ml of DMEM. The flasks were then incubated in the 5% CO_2 incubator at 37℃ to allow the attachment of cells.

3.5.3(b) Sub-culturing of cells

When the cell reached 80% confluence the cells were passaged. The process was done by removing the medium from the flask and rinsed with 2ml of PBS. The PBS was discarded followed by addition 1ml of Trypsin-EDTA solution into the 75cm^2 cell culture flask, and left in the 5% CO_2 incubator for 5 minutes for the cell to detach. The flask was gently shaken to make sure the cell was fully detached, and then the flask was observed under the inverted microscope. Three milliliter of fresh medium was added to the flask together with the trypsin, and then transferred into the centrifuge tube. The tube was then centrifuged at 1000rpm for 5 minutes. The supernatant was removed and the cell

pellet was resuspended with 1ml medium and divided into three new flasks. About 7ml of fresh medium was added to each 75cm^2 and the flask was incubated.

3.5.3(c) Determination of cell number for seeding

3.5.3(ci) Trypan blue 0.1%(w/v) solution for cell counting

The solution (10ml) was prepared by diluting 2.5ml of readymade 0.4 %(w/v) Trypan blue (Gibco BRL, UK) in 7.5ml PBS and kept at room temperature until use. About 10µl of cell suspension was added with 90µl of Trypan blue solution. The mixture was resuspended in 1.5ml eppendorf tube. Then 10µl of the mixture was pipetted onto the haemocytometer (Figure 3.1) and observed under inverted microscope. The number of viable cell estimated as below:

$$\text{Number of viable cells/ml} = \frac{\text{n x dilution factor x } 10^4}{4}$$

n = Total number of stained cell in 4 grid

The number cell that counted for seeding in 96 well plate was 1x10^4

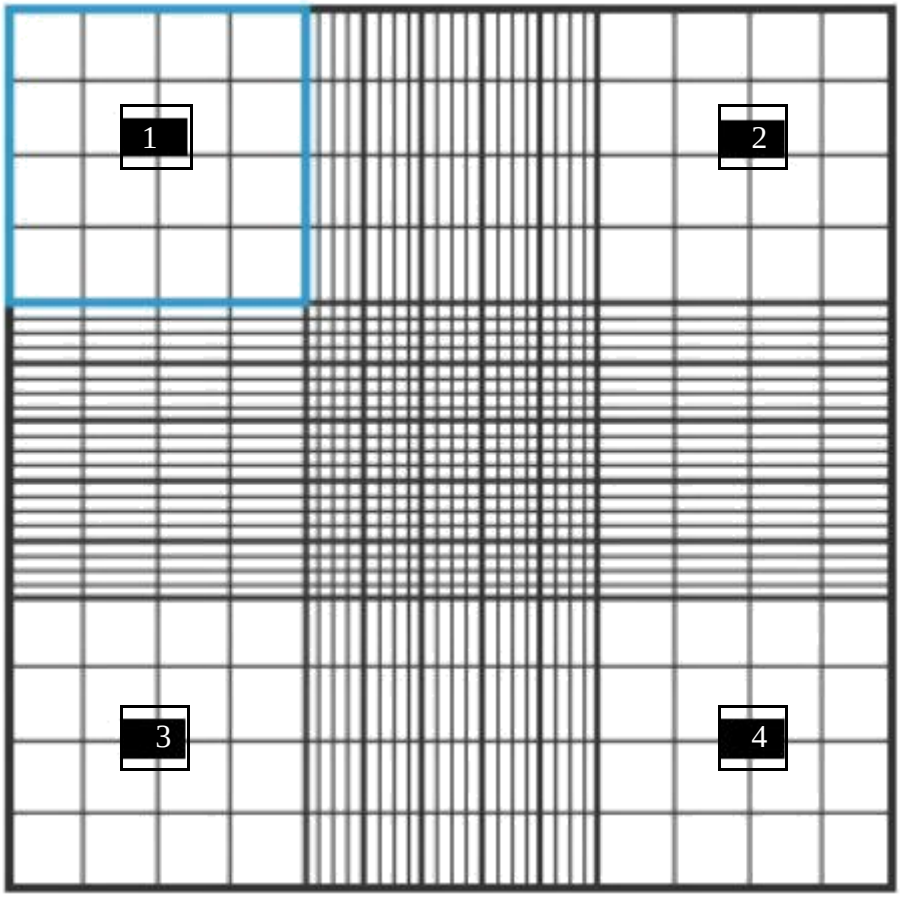

Figure 3.1: Illustration of haemacytometer

The numbered area are the area which cell were counted under inverted microscope

http://a.static-abcam.com/CmsMedia/Media/haemocytometerdiagramv3.jpg

3.6 Preparation of bee pollen extract

The stock solution of bee pollen extract was freshly prepared by diluting 0.1g of bee pollen extracts with 1ml DMEM with the final concentration of 100mg/ml, and then filtered using 0.2µl sterile filter unit (Millipore,USA). Stock solution was further diluted to desire concentration (2.5mg/ml, 5mg/ml, 10mg/ml, 20mg/ml and 40mg/ml).

3.6 Preparation of cisplatin

The stock solution of 10mM cisplatin was prepared by dissolving 6mg of cisplatin (Trevigen,USA) (MW= 300.01 g/mol) with 2ml DMEM. The solution was well mixed and aliquoted into several sterile microcentrifuge tubes and stored at -20℃ . The working solutions of the drug were freshly prepared in assay medium for prior usage.

3.7 Cytotoxicity determination using MTT assay

The Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific, USA) was used to determine the anti-proliferative effect of cisplatin and bee pollen extract, as a single agent or in combination treatment on L929 and MCF-7 cells. MTT assessing cell metabolic activity of NAD(P)H-dependent cellular oxidoreductase enzymes, in which under defined conditions reflect the number of viable cells. NAD(P)H-dependent oxidoreductase enzymes largely present in the cytosolic compartment of the cell.

3.7.1 Treatment controls

The following controls were prepared in each experimented setup in order to calculate the percentage of anti-proliferative activity of bee pollen extract and cisplastin:

a) Background/blank control

One well contained 200µl of assay medium alone or DMSO

b) Negative control

Control well contained untreated cells.

3.7.2 Treatment of cell using MTT assay

3.7.2(a) Treatment with bee pollen extract and cisplatin alone

Cells were harvested and counted as described in 3.5.3(c) and seeded about 1 x 10^4 cells in 96 wells plate. The plate was incubated overnight in the incubator with humidified atmosphere containing 5% CO_2 at 37℃. After incubated overnight the growth medium was carefully removed when the cell reached 80% confluence. Then 200µl of freshly prepared assay medium containing different concentration bee pollen extract which were (2.5mg/ml, 5mg/ml, 10mg/ml, 20mg/ml and 40mg/ml). Similarly for cisplastin treatment, the cells were treated with different concentrations of cisplatin at 2.5 µM, 5µM, 10µM, 15µM, 20µM, 30µM, 40µM and 50µM. Each experiment was done in triplicate. Once treated, the 96 well plate was incubated for 48 hours.

3.7.2(b) Treatment with bee pollen extract in combination with cisplatin

Cells were harvested, counted before being seeded in 96 wells plate as described in 3.5.3(c). The stock culture was counted for $1X10^4$ cells/ml then seeded 200µl triplicate, for bee pollen extract drug combination with cisplatin in 96 well plate. The plate then incubated with humidified atmosphere containing 5% CO_2 at 37℃. When the cell reached 80% confluence, the growth medium was carefully removed. For combination treatment about 200µl fresh prepared medium containing with (2.5µM, 5µM, 10µM, 15µM, and 20µM) with constant concentration of 15mg/ml bee pollen extract. Once treated, the 96 well plate was incubated for 48 hours. Each experiment was done in triplicate. The data from of drug combination was analysed using Compusyn software to determine the synergistic, additive and antagonistic effect of these 2 drugs combination.

3.7.2(c) Preparation of MTT assay reaction mixture

Twelve millimolar MTT stock solution was prepared by adding 1mL of sterile PBS to 5mg of MTT. The mixture was mixed by vortexing or sonicating until it dissolved. The medium which contain L929 or MCF-7 cells was then removed and replaced with 100 µL of fresh culture medium. 10µL of the 12mM MTT stock solution was added to each well, and then the cells was incubated at 37°C for 4 hours. After labeling the cells with MTT, the medium was removed but with only 25µL of medium left from the wells. About fifty microliter of DMSO (Sigma Aldrich, USA) was added to each well and mixed thoroughly with the pipette. The plate was incubated at 37°C for 10 minutes and the absorbance was read at 540 nm. The percentage of cell proliferation was calculated using formula below:

The percentage of cell proliferation was calculated using formula below:

$$\% \text{ cell proliferation} = \frac{\text{Absorbance sample - Absorbance blank}}{\text{Absorbance negative – Absorbance blank}} \times 100$$

3.8 Statistical analysis

All value was expressed as the mean $\pm$ standard deviation of triplicate analyses. Due to the sample size was <30, all statistical analysis in this study was determined using non parametric independent T test using IBM SPSS statistics version 18 (IBM Inc, USA).